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Research paper

Immunosensor for bovine anaplasmosis diagnosis on graphite platform functionalized with poly (3-hydroxybenzoic acid)

Imunossensor para diagnóstico da anaplasmose bovina em plataforma de grafite funcionalizada com poli(ácido 3-hidroxibenzóico)

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ABSTRACT*:* This study describes the development of an electrochemical immunosensor with a transducer platform functionalized with the poly-(3- hydroxybenzoic acid), to detection of antibodies against the surface protein (Am1) of *Anaplasma marginale*. This biological probe was immobilized on the polymer under graphite and characterized electrochemically. The detections of antigen-antibody interactions were conducted using the signal obtained from the oxidation of 4-aminoantipyrine (4 -AAP), interleaved with Am1, to enable the use of voltammetry technique of differential pulse. The detections of antibodies of A. marginable, using the graphite electrode functionalized with the probe Am1 was 66% higher than the graphite electrode not functionalized. The antigen (Am1) interacted with positive serum in dilutions of 1:1000, 1:800, 1:400, 1:200, 1:100, 1:50, and 1:25, demonstrating excellent sensitivity of the immunosensor. The time of storage of the immunosensor was satisfactory, with reduced peak current by 35%, after 90 days. The results demonstrate the excellent applicability of this new functionalized platform for probe immobilization and diagnosis of anaplasmosis without the interference of the most similar bovine diseases.

Keywords: Electrochemical. Voltammetry. Immunosensor. *Anaplasma marginale*. Surface modification.

RESUMO*: Este estudo descreve o desenvolvimento de um imunossensor eletroquímico com plataforma transdutora funcionalizada com opoli (ácido 3-hidroxibenzóico) para detecção de anticorpos contra a proteína de superfície (Am1) de Anaplasma marginale. Esta sonda biológica foi imobilizada no eletrodo de grafite funcionalizado com o polímero e caracterizada eletroquimicamente. As detecções das interações antígeno-anticorpo foram realizadas utilizando-se o sinal obtido a partir da oxidação da 4-aminoantipirina (4-AAP), intercalada com Am1, para possibilitar o uso da técnica de voltametria de pulso diferencial. A detecção de anticorpos de A. marginale, utilizando o eletrodo de grafite funcionalizado com a sonda Am1 foi 66% superior em relação ao eletrodo de grafite não funcionalizado.O antígeno (Am1) interagiu com o soro positivo nas diluições de 1:1000, 1:800, 1:400, 1:200, 1:100, 1:50 e 1:25, demonstrando excelente sensibilidade do imunossensor. O tempo de armazenamento do imunossensor foi satisfatório, com redução da corrente de pico em 35%, após 90 dias. Os resultados demonstram a excelente aplicabilidade desta nova plataforma funcionalizada para imobilização de sondas e diagnóstico de anaplasmose sem a interferência das doenças bovinas mais semelhantes.*

Palavras-chave: *Eletroquímica. Voltametria. Imunossensor. Anaplasma marginale. Modificação de superfície.*

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INTRODUCTION

The efficient production of livestock in tropical and subtropical regions worldwide is severely impaired by endemic hemoparasitic diseases including anaplasmosis, babesiosis, heartwater, theileriosis and trypanosomiasis (PALMER et al., 1987). *Anaplasma marginale*, a tick-borne pathogen of cattle, infects erythrocytes and causes the disease anaplasmosis. The acute phase of anaplasmosis is characterized by anemia, fever, weight loss, reduced milk production and often death contributing to significant economic loss to the dairy and meat industries (ESTEVES et al., 2009).

Diagnostics are crucial for identifying the presence and cause of disease at the individual and population levels, both in humans and animals. In addition, timely detection of infectious agents is critical in early diagnosis and treatment of infectious diseases. The development of rapid, accurate, and portable diagnostic tools is very important because infectious diseases are a major global-health and economic burden, especially in resource-limited settings (LOPEZ et al., 2001).

Anaplasma marginale is usually diagnosed by enzyme-linked immunosorbent assay ELISA (OKAFOR et al., 2019), card agglutination (FOSGATE et al., 2010), indirect fluorescent antibody test (SILVEIRA et al., 2016), complement fixation test (COETZEE et al., 2007); radioimmunoassay (SCHUNTNER; LEATCH, 1988) and polymerase chain reaction (PCR) (CARELLI et al., 2007; DE SOUZA RAMOS et al., 2019). The performance and applicability of ELISA, radioimmunoassay and PCR require relatively sophisticated infrastructure, well-equipped laboratories and must be performed by highly trained staff (CORTINA et al., 2016).

In this direction, biosensors for infectious diseases diagnosing have emerged. Biosensors have found many applications in every sphere of life. They find their use in diagnosis (BAHADIR; SEZGINTÜRK, 2015; LABIB; SARGENT; KELLEY, 2016; TARASOV et al., 2016; WANG et al., 2014; NIAMH; RYONA SAYERS; ALAN O'RIORDAN, 2015; ZAKIAN et al., 2017; WU et al., 2018), food technology [(ROTARIU et al., 2016;MADURAIVEERAN; JIN, 2017; VARMIRA et al., 2018; SANI et al., 2018), biotechnology (TIWARI et al., 2016;YING et al., 2018;KIM et al., 2019; SHETTI et al., 2019), engineering (REDDY et al., 2011; GU et al., 2010; ABDULBARI; BASHEER, 2017; (ROGERS; TAYLOR; CHURCH, 2016), environmental monitoring (ARDUINI et al., 2017; CAMPOS-FERREIRA et al., 2013; SGOBBI; MACHADO, 2018) among others.

Electrochemical sensing strategies have the potential to achieve rapid, sensitive, selective and low-cost detection of biomolecular analytes relevant to clinical diagnosis and monitoring treatment of disease (LABIB; SARGENT; KELLEY, 2016;HUANG et al., 2017; BLAIR; CORRIGAN, 2019). Other advantages include simple and low-cost instrumentation, of both potentiostats and disposable mass-produced electrodes (TALEAT; KHOSHROO, 2014).

Recognition elements are central in the biosensor performance and selectivity toward a particular analyte. They are currently used in the preparation of electrochemical sensors for clinically relevant biomolecules include antibodies, antibody fragments, enzymes, receptors, lectins, whole cells, peptides/proteins, nucleic acids, aptamers, peptide nucleic acids, locked nucleic acids, and molecularly imprinted polymers (LABIB; SARGENT; KELLEY, 2016).

The implementation of advanced materials, which are suitable for selective interaction with analyte and/or conversion of this interaction into analytical signal, offers new opportunities in sensors and biosensors. Conducting polymer based electrochemical

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biosensors have gained great attention as such biosensor platforms are easy and cost-effective to fabricate, and provide a direct electrical readout for the presence of biological probes with high sensitivity and selectivity (ADHIKARI; MAJUMDAR, 2004; PALLELA et al., 2016).

Conducting polymer materials themselves are both sensing elements and transducers of the biological recognition event at the same time, simplifying sensor designs. Electrochemical biosensors fabricated using conducting polymers are highly stable and ultrasensitive because biomolecules can be covalently immobilized on the polymer backbone possessing COOH or NH_2 groups (PALLELA et al., 2016).

In this work, we develop an electrochemical immunosensor for diagnosing bovine infections caused by *Anaplasma marginale* using a graphite platform functionalized with poly(3-hydroxybenzoic acid). The performance of the immunosensor was evaluated using cyclic and pulse differential voltammetry. To assess the clinical usefulness of the sensor, we conducted direct detection of *Anaplasma marginale* in serum samples.

MATERIAL AND METHODS

Chemicals

All reagents were of analytical grade and used as received. Ultrapure water (Millipore Milli-Q system) was used in the preparation of the solutions. Solutions of 3-HBA (2.5 $mmolL^{-1}$) were prepared in $HClO_4$ solution (0.5 $molL^{-1}$) immediately before use. All essays were conducted at room temperature (25 ± 1ºC).

Apparatus

The electrochemical experiments were carried out in a three-compartment Pyrex glass cell provided with three electrodes and degassing facilities for bubbling N_2. As working electrode was used a graphite disc with area of 1.0 cm^2 (99.9 %, Alfa Aesar). The reference system was Ag/AgCl (KCl 3.0 mol L^{-1}) and all potentials are referred to this system. The auxiliary electrode was a 2cm^2 Pt foil. The electrochemical experiments were also performed using a CH Instruments model 420A potenciostat. Prior to electro polymerization, the graphite surface was mechanically polished with alumina slurry (0.3 μm diameter), sonicated, washed with distilled water and dried in the air. All solutions were degassed by nitrogen bubbling.

Electro polymerization of the 3-HBA

The growth of poly(3-HBA) were conducted on graphite electrodes (100 successive scans of potential, between 0.0 and 1.2 V *vs*. Ag/AgCl, at 50 mVs^{-1}) using 3-HBA solution (2.5 $mmol.L^{-1}$), prepared in $HClO_4$ (0.5 $mol.L^{-1}$) [(FERREIRA et al., 2014)] .

Procedure for the construction of the immunosensor for the diagnosis of bovine anaplasmosis

The physicochemical transducer was developed from the immobilization of a specific surface protein of *Anaplasma marginale* obtained from the sequence STSSQLGGGSSTSSQLGGGSSTSSQL (*Am1*). Therefore, the biological material was

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design and chemically synthetized by GenScript EUA Inc., linked in BSA to increase the probe antigenicity.

After electro polymerization, the modified electrode was washed vigorously with deionized water and 18 µL of *Am1* in medium of acetate buffer (pH = 4.3) was dropped onto the surface of electrode. The concentration of Am1 used was 0.055 µg µL^{-1}. Then the electrodes were dried at 37 °C for 15 minutes. Afterwards, the electrode was washed with phosphate buffer (pH 7.3), to remove the peptides did not interact with the electrode surface.

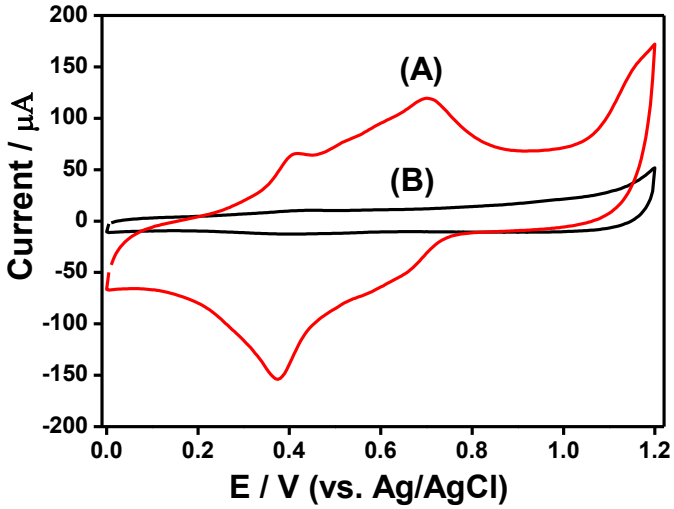
Then, 18 µL of positive (IgG +) or negative (IgG-) serum diluted in phosphate buffer was added to the electrode. These electrodes were kept in an oven at 37° C for 1.500 seconds. Then the electrodes were washed with phosphate buffer to remove serum that did not interact with *Am1.* After the excess serum was withdrawn, 18 µL of 4-aminoantipyrine (10 mmol L^{-1}) was added to the electrodes.

The drying and washing procedure was repeated. Blood samples from healthy animals (IgG-) and contaminated with *A marginale* (IgG +) tested in this work were donated by Desidério Finamor Veterinary Research Center, Eldorado do Sul, RS–Brazil. Serum samples from infected animals with *A. margianale* was diluted in phosphate buffer (1: 1000, 1: 800, 1: 400, 1: 200, 1: 100, 1:50, 1:25). All measurements were performed in triplicate.

RESULTS AND DISCUSSION

The electrochemical profile of graphite electrode modified with poly(3HBA) in acidic medium showed current peaks at 0.44 V and 0.68 V (oxidation peaks), and 0.43 and 0.60 V (reduction peaks), *vs*. Ag/AgCl, suggesting a reversible behavior (**Figure 1**).

Figure 1. Cyclic voltammograms obtained in medium of perchloric acid (0.5 mol L^{-1}) for (A) graphite electrode and (B) poly (3-HBA) modified graphite electrode, v = 50 mV s^{-1}



The poly(3-HBA) film remained stable on the graphite electrode surface for successive cycles in medium of perchloric acid, such as previously reported in the literature (FERREIRA et al., 2014). After electro polymerization the electrode was washed with deionized water and the immobilization of *Anaplasma marginale* (Am1) on the surface electrode was investigated. The immobilization of biomolecules on the electrode surface plays an important role in the sensitivity, selectivity and stability of the

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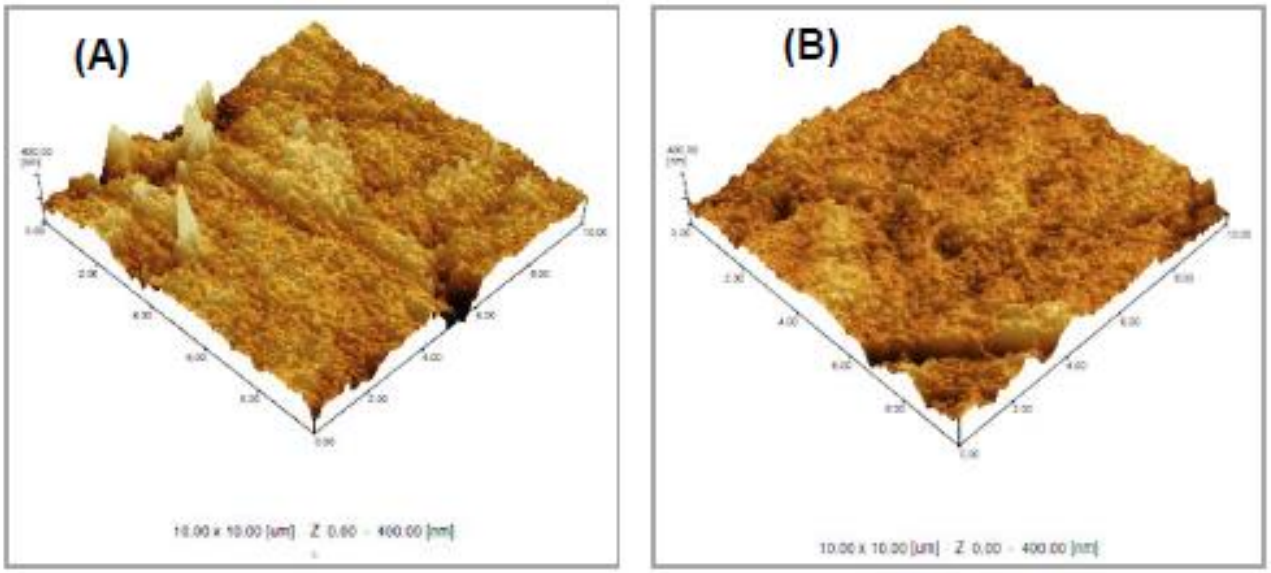
immunosensor(AYDEMIR et al., 2017; COSNIER, 1999; KUCHERENKO et al., 2016; TANG et al., 2013).

The **Figure 2** shows the atomic force microscopy images (AFM) from graphite electrode (**Figure 2A**) and poly (3-HBA) modified graphite electrode (**Figure 2B**).

Figure 2. Atomic force microscopy images from (A) graphite electrode and (B) poly (3-HBA) modified graphite electrode



Visually, it can be noticed that the surface of the graphite electrode (Figure 2A) presents a greater number of valleys and peaks (more irregular surface and porous) in relation to the modified surface of the graphite electrode after electro polymerization in 10 potential sweep cycles (Figure 2B). The roughness values determined by AFM for the graphite electrode was 98.66 ± 5.6 nm and modified with poly(3-HBA) was 24.5 ± 1.87 nm. The roughness values show that the film reduced by approximately 75% the roughness of the graphite electrode surface.

This considerable reduction in the roughness of the graphite electrode modified with a thin polymeric film, electropolymerized only with 10 potential cycles, is due to the three-dimensional structure of poly (3-HBA)(FERREIRA et al., 2014).

Thus, *Anaplasma marginale* (*Am1*) was immobilized on the poly (3HBA) modified graphite electrode and its indirect detection by differential pulse voltammetry was evaluated.

It can be observed that 4-aminoantipyrine (4-AAP) allowed differentiate between positive serum detections (**Figure 3a**) from blank (**Figure 3b**) and negative serum (**Figure 3c**).

Figure 3a shown that 4-AAP interact with both Am1 and the antibody increasing peak current. The increase of peak current is related to the amount of electroactive interleaver on the electrode surface, due to *Am1* is interacting with the IgG^+ (macromolecule) of animals infected *with A. marginale*. These experiments showed that the analytical signal obtained decreased in the presence of negative serum and blank (**Figure 3b and 3c**). It was found that immobilization of *Am1* and detection of antibody (IgG^+) on the poly (3-HBA) functionalized graphite electrode showed an increase of approximately 70% in peak current compared to the non-functionalized electrode under the same conditions (**Figure 4**).

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Figure 3. Differential pulse voltammograms of the poly (3-HBA) modified graphite electrode after addition of **(a)** 18 µL of Am1 (0.055 µg µL^{-1}) + 18 µL of positive serum (1:25 dilution) + 18 µL 4-AAP at 10 mmol L^{-1}; **(b)** 18 µL of Am1 (0.055 µg µL^{-1}) + 18 µL 4-AAP at 10 mmol L^{-1}; **(c)** 18 µL Am1 (0.055 µg µL^{-1}) + 18 µL negative serum (1:25 dilution) + 18 µL 4-AAP at 10 mmol L^{-1}. Electrolyte: 0.1 mol L^{-1} phosphate buffer, pH 7.3. Experimental conditions: Amplitude: 25 mV; pulse duration 60 ms; v = 10 mV s^{-1}

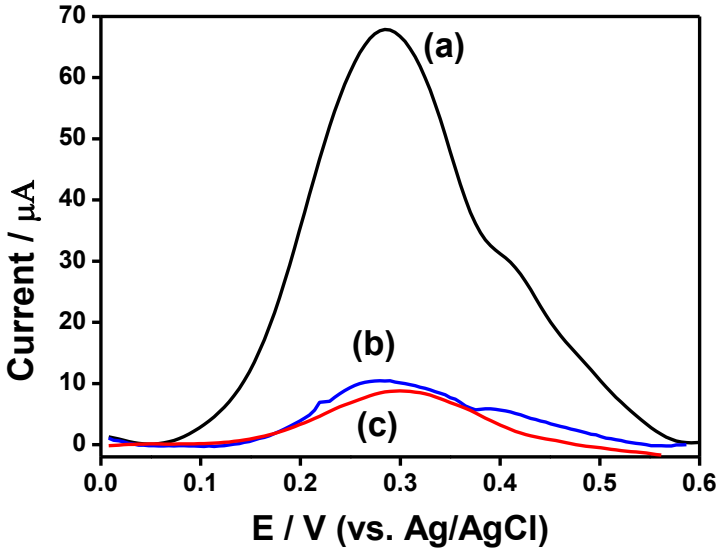
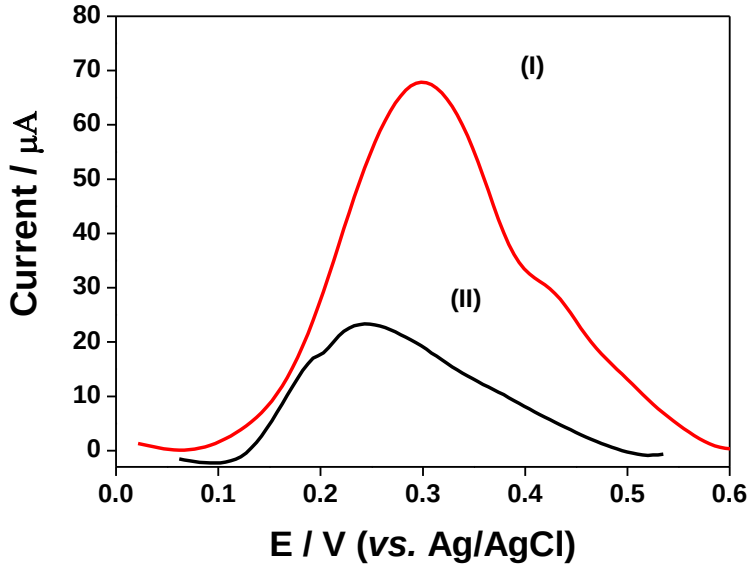


Figure 4. Differential pulse voltammograms for: (I) poly (3-HBA) modified graphite electrode after addition of 18 µL Am1 (0.055 µg µL^{-1}) + 18 µL positive serum (1:25 dilution) + 18 µL 4-AAP at 10 mmol L^{-1}; (II) unmodified graphite electrode with film after addition of 18 µL Am1 (0.055 µg µL^{-1}) + 18 µL positive serum (1:25 dilution) + 18 µL 4-AAP at 10 mmol L^{-1}. Electrolyte: 0.1 mol L^{-1} phosphate buffer, pH 7.3. Experimental conditions: Amplitude: 25 mV; pulse duration 60 ms; v = 10 mV s^{-1}



Thus, the poly(3HBA) functionalized graphite electrode provided an increased sensitivity in the detection of nitrogenous bases and the complementary target of Am1.

Direct detection of Am1 was not possible on the functionalized platform because of the 20 amino acids presents in proteins only tyrosine, tryptophan, histidine, cysteine and

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methionine are electroactive on carbon electrodes (ENACHE; OLIVEIRA-BRETT, 2013) and none of them are present in the structure of Am1.

The effect of 4-AAP concentration on peak current was evaluated. In quantitative terms, the data on current response showed the best values to be those obtained using 10mmol L^{-1} of 4-AAP. This finding led to the choice of 10 mmol L^{-1} concentration for the analytical determinations (indirect detections).**Figure 5** shows immobilization of Am1 on the functionalized electrode at different times.

Figure 5. Differential pulse voltammograms for poly (3-HBA) modified graphite electrode after addition of 18 µL of Am1 (0.055 µg µL-1) + 18 µL 4-AAP at 10 mmol L-1 + 18 µL of positive serum (1: 100 dilution) as a function of the interaction time of Am1 with the specific antibody (300 seconds, 600 seconds, 900 seconds, 1200 seconds, 1500 seconds and 1800 seconds). Electrolyte: 0.1 mol L^{-1} phosphate buffer, pH 7.3. Experimental conditions: Amplitude: 25 mV; pulse duration 60 ms; v = 10 mV s^{-1}

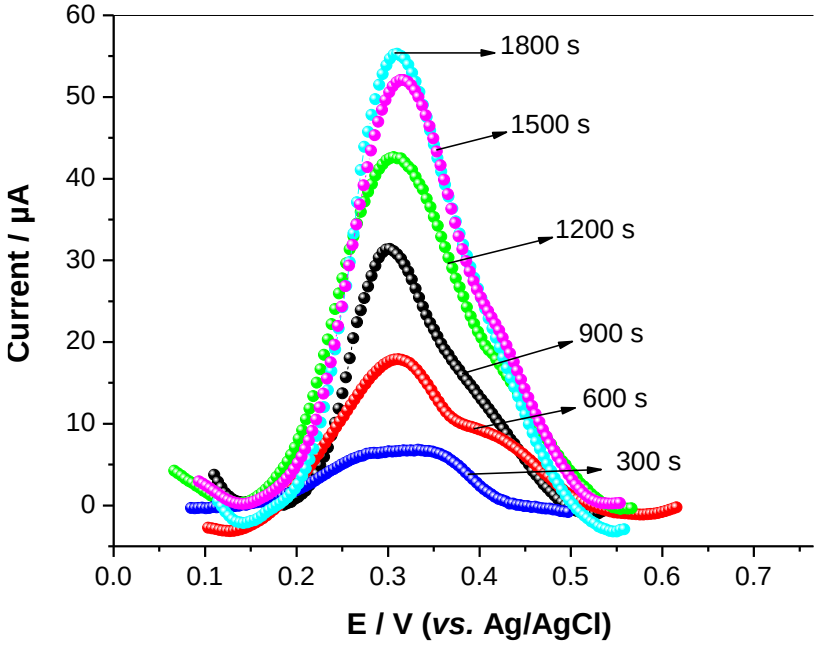

Figure 5 shows an increase in current values from 300 to 1800 seconds of interaction (at 37°C). After 1.500 seconds the current responses did not change significantly. Therefore, 1.500 seconds was chosen as optimal for the rest of the experiments.

These results were attributed to the increased electron density on the surface electrode because it was preconditioned by chronoamperometry (-0.2 V for 120 s). Electrostatic interactions as well as dipole ion interaction can occur between the polymeric film and the peptide, which was diluted in acetate buffer pH = 4.3. At this pH value the peptide has a positive residual charge (PI = 5.8).

As already mentioned, serum samples refer to samples from animals infected with *A. marginale.* In this work the IgG^{+} content was not quantified in the samples because the serum of these animals is a complex sample with several immunoglobulin types (IgGs). For calibration curve assays the positive serum from animals infected with *A. marginale* was diluted in phosphate buffer at pH 7.3 in the following proportions: 1: 1000; 1: 800; 1: 400; 1: 200; 1: 100, 1; 50, 1:25, using the constant concentration of Am1 (0.055 µg $µL^{-1}$). **Figure 6** shows peak currents as a function of successive dilutions of positive serum samples.

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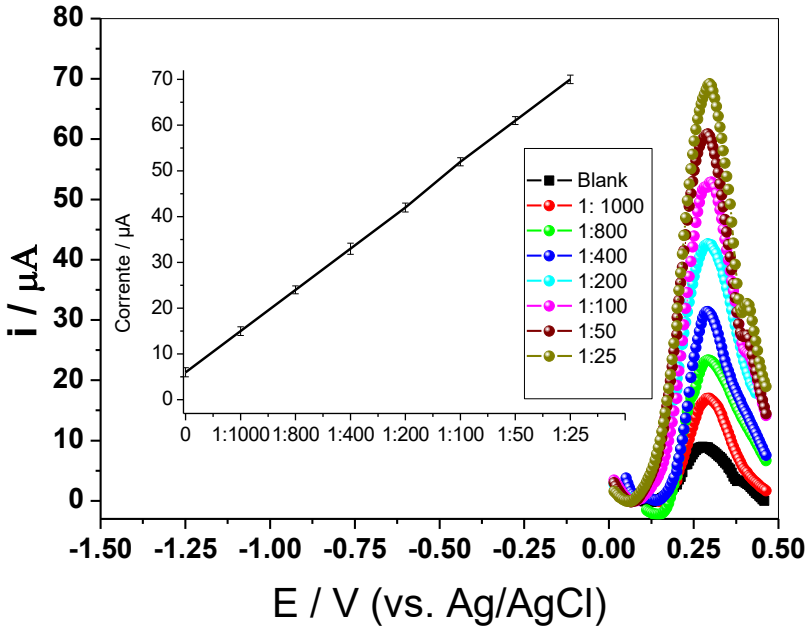
Figure 6. Differential pulse voltammograms for poly (3-HBA) modified graphite electrode after addition of 18 µL of Am1 (0.055 µg μL^{-1}) + 18 µL 4-AAP at 10 mmol L^{-1}, with addition of 18 µL of anaplasmosis bovine serum after the following dilutions: 1: 1000; 1:800; 1:400; 1:200; 1:100, 1:50, 1:25. In all measurements, the blank sign was subtracted. Electrolyte: 0.1 mol L^{-1} phosphate buffer, pH 7.3. Experimental conditions: Amplitude: 25 mV; pulse duration 60 ms; v = 10 mV s^{-1}



Linear current regression analysis (µA) versus the dilutions (1: 1000; 1: 800; 1: 400; 1: 200; 1: 100, 1; 50, 1:25) of anaplasmosis bovine serum (**Figure 6**) showed excellent linearity (r> 0.99). The development of an immunosensor for the diagnosis of bovine anaplasmosis with good sensitivity is indispensable, since animals infected with *A. marginale* often become persistently infected serving as reservoir (SINGH et al., 2012).

Clinical signs of anaplasmosis are not specific and serological diagnosis is required (VIDOTTO; MARANA, 2001).

The molecular recognition in the samples by the biological component is fundamental in the development of immunosensors. Therefore, the analysis of interferents is necessary to obtain reliable results. The interferents were added along with the positive serum from an *A. marginale* infected animal in the presence of a disease that normally affects the animals concomitantly with bovine anaplasmosis. Positive serum detections for bovine anaplasmoses in the presence of antibodies of some diseases such as brucellosis, *B. bovis* and *B. Bigemina* (Bovine parasitic sadness) are presented in **Figure 7**.

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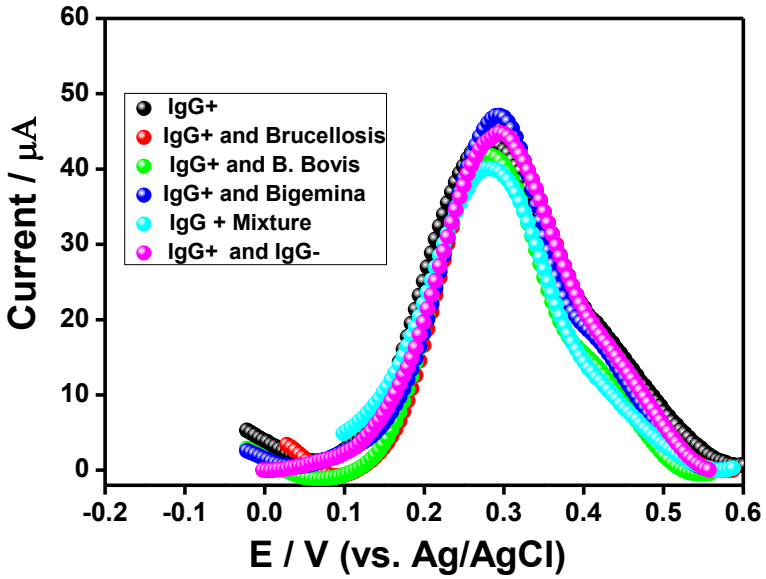
Figure 7. Differential pulse voltammograms for poly (3-HBA) modified graphite electrode after addition of 18 µL of Am1 (0.055 µg μL^{-1}) + 18 µL 4-AAP at 10 mmol L^{-1}, with addition of 18 µL from anaplasmosis (1: 100) bovine serum mixture with 1: 100 positive dilution serum to: *Brucellose, B. bovis, B. bigemina* and Mix (mixture of all interfering sera). The negative serum was diluted (1: 100). In all measurements, the blank sign was subtracted. Electrolyte: 0.1 mol L^{-1} phosphate buffer, pH 7.3. Experimental conditions: Amplitude: 25 mV; pulse duration 60 ms; v = 10 mV s^{-1}



No influence of interferents on the voltametric response was observed, therefore, the sensor showed good selectivity. Immunosensor storage time was evaluated for 90 days. Between evaluations, the electrode was stored in a hermetically sealed vial in the freezer until the next detection. It is worth mentioning before being stored the electrode was functionalized with poly (3-HBA) and immobilized with membrane protein Am1. At the time of analysis, positive serum (dilution 1:25) and intercalator (10 mmol L^{-1}) were added, the interaction time was 1.500 seconds. The electrolyte used was phosphate buffer (pH 7.3). The electrochemical response *versus* immunosensor storage time is shown in **Figure 8**.

After 30 days the immunosensor showed a current decrease of approximately 3% and 30% in 90 days. The immunosensor exhibits good stability.

In work carried out by Brahim et al. (2002), the development of an amperometry biosensor with immobilization of the glucose oxidase enzyme, in hydrogel film functionalized with polypyrrole, At the end of ninety days, it showed a 30% drop in response, the limit value accepted for commercialization of the biosensor.

The good stability presented by the immunosensor developed for the diagnosis of *A. marginale* is related to the stable structure of the membrane protein (Am1, oligopeptide with only 26 amino acid residues) immobilized on the EG / poly (3-HBA) sensor surface. This stability is one of the indispensable factors for this immunosensor to be marketed mainly for continuous monitoring applications. Tasuma (1992) states that exposing immunosensor to air can cause a decrease in sensitivity by deactivating the biomolecules or by oxidation of the polymeric material used in the functionalization of the surface sensor.

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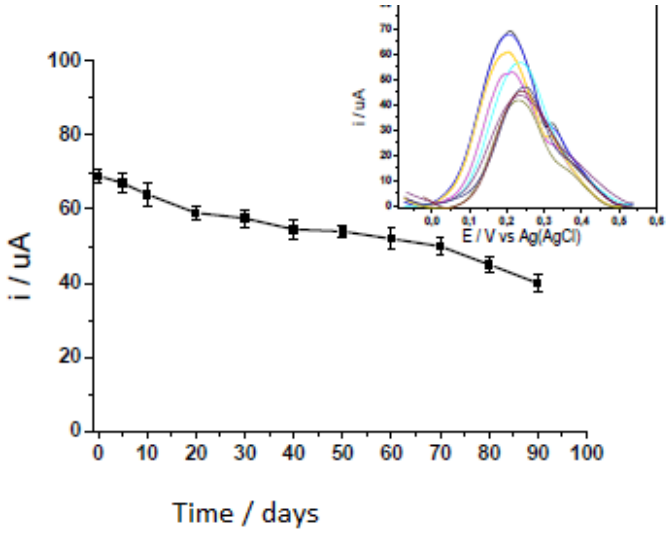
Figure 8. Peak current *versus* storage time (in days) from imunossensor



CONCLUSIONS

The anaplasmosis immunosensor from bovine serological samples was built on the poly (3-HBA) platform. Detections were performed by signal obtained by oxidation of the 4-AAP protein interleaver using the differential pulse voltammetry technique.

The immunosensor showed good sensitivity, antigen (Am1) interacted with positive serum in dilutions of 1:1000, 1:800, 1:400, 1:200, 1:100, 1:50, 1:25 (with excellent linearity).

The main contribution of this work to the international literature was the development of an immunosensor to identify anaplasmosis in real samples the serum cattle. This biomonitoring, in real time, will be very important for the diagnosis of bovine anaplasmosis in beef herds and dairy since this disease causes severe damage to livestock in our country.

As future proposals for the immunosensor, the system needs to be miniaturized and applied in economic feasibility studies for commercial deployment.

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